INVESTIGATION OF IMMUNOMODULATION ON MYOFIBROBLAST ACTIVATION: IMPLICATIONS FOR FIBROTIC DEVELOPMENT IN WOUND HEALING

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INVESTIGATION OF IMMUNOMODULATION ON MYOFIBROBLAST ACTIVATION: IMPLICATIONS FOR FIBROTIC DEVELOPMENT IN WOUND HEALING

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LIST OF SYMBOLS AND ABBREVIATIONS

ECM	Extracellular matrix
TGF-β	Transforming growth factor-beta
α-SMA	Alpha-smooth muscle actin
M1	Inflammatory macrophage phenotype
M2	Anti-inflammatory macrophage phenotype
M2a	Wound healing macrophage subtype
M2c	Regulatory macrophage subtype
IFN- γ	Interferon-gamma cytokine
TNF- α	Tumor necrosis factor-alpha
IL-4	Interleukin-4 cytokine
IL-10	Interleukin-10 cytokine
LPS	Lipopolysaccharide

SUMMARY

Endogenous mechanisms of wound healing and remodeling are a particularly attractive avenue for targeting traumatic injury and incomplete growth. Macrophages are highly involved in this process, and generally exhibit either an inflammatory (M1) phenotype, promoting cell debris clearance, or an anti-inflammatory (M2) phenotype, promoting tissue regeneration and remodeling, although fluidity exists in these phenotypes for injury repair in vivo. Type I collagen is also crucial to the repair process through development of extracellular matrix (ECM), which provides scaffolding for cellular and vascular growth as well as controlling cell differentiation later in the process. Myofibroblasts are the source of Type I collagen deposition, and differentiate from fibroblasts in the presence of transforming growth factor-beta (TGF-β), among other pathways. However, persistent myofibroblast activity can perpetuate fibrosis, leading to incomplete repair and scarring. While it is clear that macrophages and myofibroblasts are involved in the wound healing process, the interplay between these two populations has not been thoroughly investigated. Two in vitro experiments of M1, M2a, and M2c macrophage phenotypes with 10T1/2 fibroblasts were conducted. The first experiment involved fibroblasts interacting with cytokines produced by each macrophage phenotype at three different initial seeding densities: 500,000, 750,000, and 1 million cells per well. The second experiment involved fibroblasts and macrophages in a co-culture. Expression of alpha-smooth muscle actin (α-SMA), an indicator of myofibroblast activation, was probed via immunofluorescence after 72-hour incubation for both experiments. Through confocal microscopy and image analysis, it was determined that fibroblast interaction

with M1 soluble factors lead to significantly higher levels of normalized α-SMA expression within fibroblasts compared to the M2a and M2c at a seeding density of 750,000. However, the co-culture model, with macrophages of different phenotypes interacting with fibroblasts in a contact dependent manner, saw no significant difference between these groups or the control. M2a and M2c may play an important role in promoting tissue regeneration over excessive collagen production and scar tissue development, whereas pro-inflammatory signaling from M1 may promote more collagen production. However, the lack of significant results from contact dependency may suggest different macrophage behavior. More investigation is necessary to comprehensively understand macrophage-fibroblast/myofibroblast interplay in wound healing.

CHAPTER 1

INTRODUCTION

Incomplete healing of soft tissue after traumatic injury is a major clinical burden, comprising 50-70% of combat injuries and 80% of limb amputations in soldiers (Sicari et al., 2012). Furthermore, 250,000 surgeries per year are conducted in order to correct muscle degeneration caused by rotator cuff tears (Sicari et al., 2012). The most effective method currently available for traumatic injury is autologous tissue repair, which requires multiple surgeries and has limited clinical success (Sicari et al., 2012). Therefore, there is a need for therapeutic methods which promote tissue regeneration at the site of injury.

Fibroblasts are mesenchymal cells that stabilize the extracellular matrix in homeostasis. During traumatic injury, however, these cells can activate into myofibroblasts, which play a key role in wound healing through deposition of type I collagen, providing scaffolding for cell growth and differentiation as well as angiogenesis (Brett, 2008; Phan, 2008). Myofibroblast activation is often attributed to TGF- β signaling and subsequent expression of α-SMA, although numerous signaling pathways and regulatory processes can affect differentiation (Phan, 2008).

Wound healing is also characterized by a recruitment of monocytes from blood circulation and differentiation of those monocytes into macrophages. Macrophages are generally inflammatory (M1), clearing cell debris and promoting early tissue regeneration in the beginning stages of wound healing, or anti-inflammatory (M2), being involved in the later stages of tissue regeneration and remodeling (Arnold et al., 2007). Previous research by Arnold *et al*. suggests M1 and M2 phenotypes are fluid in vivo, with

monocytes/macrophages converting from inflammatory to anti-inflammatory 2 days after injury (2007). This conversion is facilitated by the tissue environment, particularly the phagocytosis of cell debris and further reinforcement by reduction of localized necrosis (Arnold et al., 2007). There are a range of diverse phenotypes in the M2 spectrum of macrophages, which can be classified most succinctly by cytokine secretory profiles, which includes wound healing (M2a) and regulatory/anti-inflammatory (M2c) subtypes (Mosser et al., 2008; Spiller et al., 2014). In vitro, these macrophages subtypes can be elicited by treating macrophages with specific cytokine cocktails (Mosser et al., 2008; Spiller et al., 2014). In particular, M1 macrophage activation can be elicited by treatment with interferon-gamma (IFN- γ) and tumor necrosis factor-alpha (TNF- α), while M2a and M2c macrophage activation is dependent upon interleukin-4 (IL-4) from TH2 cells and granulocytes, and interleukin-10 (IL-10) from regulatory T cells (Mosser et al., 2008).

While it is clear that both myofibroblasts and macrophages are involved in wound healing, the interplay between the two cell populations has not been thoroughly investigated. In order to understand the mechanistic relationship between macrophage phenotype and myofibroblast activation, two different in vitro experiments were analyzed: (1) 10 T ½ mouse fibroblasts incubated with macrophage-conditioned media (containing cytokines), and (2) 10 T ½ fibroblasts co-cultured with macrophages.

CHAPTER 2

LITERATURE REVIEW

Myofibroblast activation can be elicited through a number of factors and pathways. Thy-1, a surface glycoprotein, has been shown to be a key regulator for the fibrogenic nature of fibroblasts (Hagood et al., 2005). Decreased Thy-1 expression leads to high expression of TGF- β, increased myofibroblast differentiation, and collagen deposition (Hagood et al., 2005). While myofibroblast activation and collagen deposition are critical for tissue remodeling in the wound healing process, high levels of myofibroblast activation can lead to excessive production of collagen and fibrosis at the injury site.

In terms of macrophage-fibroblast interaction, previous research using an in vitro co-culture model with lung fibroblasts has shown that classically activated (M1) macrophages inhibit fibrogenesis through TNF- α expression (Song et al., 2000). It was also shown that alternatively activated (M2) macrophages promote fibroblast activation and collagen production (Song et al., 2000). In vascular adventitia, macrophages activate the inducible nitric oxide synthase (iNOS) pathway which leads to type I and type III collagen deposition via adventitial fibroblasts (AFs) in vivo and in vitro (Zhang et al., 2016). Administering interleukin-1β (IL-1β) to AFs also elicited iNOS signaling, suggesting that the iNOS pathway may work through this particular factor (Zhang et al., 2016). However, neither of these studies highlight the implications of macrophage-fibroblast interaction on myofibroblast activation.

Interestingly, in the context of skeletal muscle injury, inflammatory cues from M1 macrophages promote muscle stem cell proliferation and migration, and anti-inflammatory cues from M2 macrophages have been shown to promote muscle stem cell differentiation into muscle fibers and subsequent muscle regeneration (Arnold et al., 2007; Saclier et. al., 2013). Studies have also shown that the absence of these macrophage causes incomplete membrane repair, muscle regeneration, satellite cell differentiation, and increased fibrotic activity during the healing process (Novak et. al, 2014; Tidball et. al, 2007).

Our lab conducts research into novel methods of regenerative medicine, including immunomodulation. In the past, it has been shown in vivo that FTY720, a Spingosine-1-receptor modulator and FDA approved drug incorporated into a biomaterial (poly lactic-co-glycolic acid (PLGA) thin film), increases M2 macrophage presence in mice spinotrapezius volumetric loss models when compared to PLGA control 3 days post-injury (San Emeterio et al., 2017). In addition, FTY720 treated mice had a higher ratio of muscle fiber to collagen volume than controls (San Emeterio et al., 2017). In particular, the collagen fibers in PLGA control animals were dense and highly aligned, which is indicative of scarring (San Emeterio et al., 2017). The effects of FTY720 in this study could be the result of a number of mechanisms, including enhanced growth and decreased collagen production. While it appears that macrophages may upregulate fibroblast activation and collagen production, macrophage-fibroblast interplay in the context of myofibroblast activation requires further investigation.

In addition, it is possible that different macrophage phenotypes secrete cytokines or participate in signaling to fibroblasts through contact dependent mechanisms that

differentially affect fibroblast activation. Previous macrophage-fibroblast in vitro research by Holt *et al*. (2010) has shown that culturing cells in the conditioned media of another cell, non-contact co-culturing (paracrine signaling) and contact dependent co-culturing (juxtacrine signaling) each yield distinct results in cellular behavior and morphology. Elucidating phenotypic-specific macrophage effects on myofibroblast activation will be important in further understanding the wound healing process, and inform the design of therapies that tune inflammatory state within an injury to achieve a more favorable outcome. Not only should the significance of macrophage phenotype on the level of myofibroblast activation be determined, but also whether contact dependency plays an important role, or if myofibroblast activation is more dependent on secreted factors from macrophages.

CHAPTER 3

MATERIALS AND METHODS

Macrophage differentiation and conditioning

In order to assess macrophage cytokine and fibroblast interaction, macrophage media conditioned with secreted cytokines of different phenotypes was prepared. A RAW 264.7 mouse macrophage cell line (ATCC® TIB-71™) was thawed from liquid nitrogen and transferred to a flask with media containing 87% Dulbecco's Modified Eagle Medium (DMEM; Gibco®), 10% Fetal Bovine Serum (FBS; Gibco®), 1% Penicillin Streptomycin (Pen Strep; Life Technologies™), 1% sodium pyruvate (Gibco®), and 1% L-glutamine (Life Technologies™). For the conditioned media experiment, macrophages were plated onto 6-well plates with 2 milliliters of macrophage media containing either 1 million, 750,000, or 500,000 macrophages per well to determine optimal seeding density for cytokine production. 24 hours after plating, the macrophages underwent phenotypic polarization through administering cytokine-specific media to each well. Wells polarized to M1 were given media with a 10 ng/mL concentration of 1:1,000 diluted lipopolysaccharide (LPS) and a 20 ng/ mL concentration of IFN- γ (PeproTech 315-05). Media for M2a and M2c wells had a concentration of 10 ng/ml IL-4 (PeptroTech 214-14) and 10 ng/mL IL-10 (PeproTech 214-10), respectively. Media from each phenotype and seeding density combination was extracted from the wells after 24 hours and transferred into 1 mL aliquots.

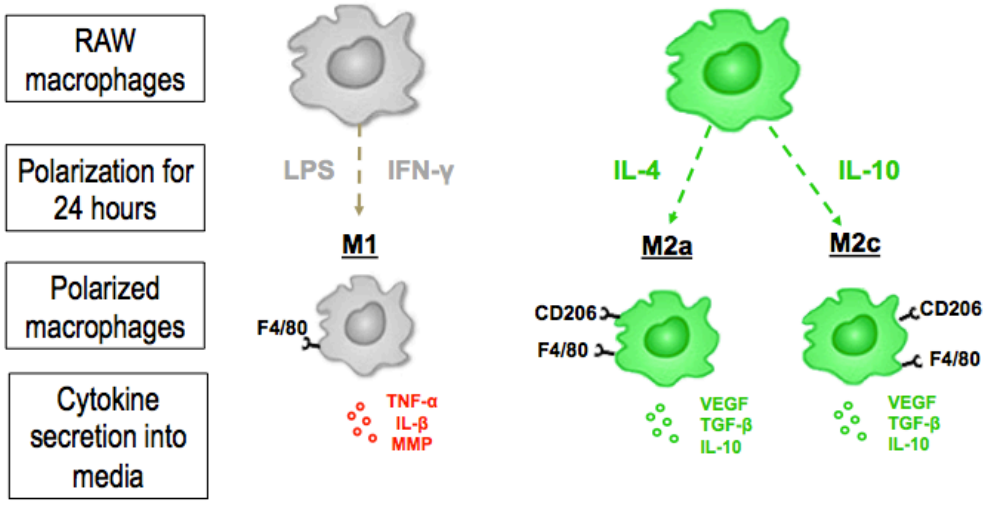


Figure 1: Macrophage Polarization and Conditioning.

Conditioned Media In Vitro Experimentation

CH3 10 T ½ fibroblasts, a clonal mouse embryonic cell line, were also thawed from liquid nitrogen at a concentration of 150,000 cells per mL and were transferred to a flask with media containing 89% Eagle's Minimum Essential Medium (EMEM; ATCC® 30-2003™), 10% FBS, and 1% Pen Strep. 10 T1/2's were then sustained until confluency was sufficient to transfer the fibroblasts to two 8-well plates. Plates contained 250 µL of media and 10,000 cells per well. 250 µL of 0.1% gelatin (Stem Cell Technologies™ #07903) was added to each well and allowed to sit for approximately 25 minutes before plating with 10 T ½'s, to allow for improved and sustained adherence. Twenty-four hours after plating, media from each of the wells was aspirated, and a combination of standard 10 T1/2 media and conditioned media with M1, M2a, or M2c polarized macrophage cytokines at different seeding densities was administered. The control well was given a total of 500 µL of 10 T ½ media and the other wells were given half (250 µL) 10 T 1/2

media and half conditioned media. The 8-well plates were incubated for 72 hours at 37 degrees Celsius.

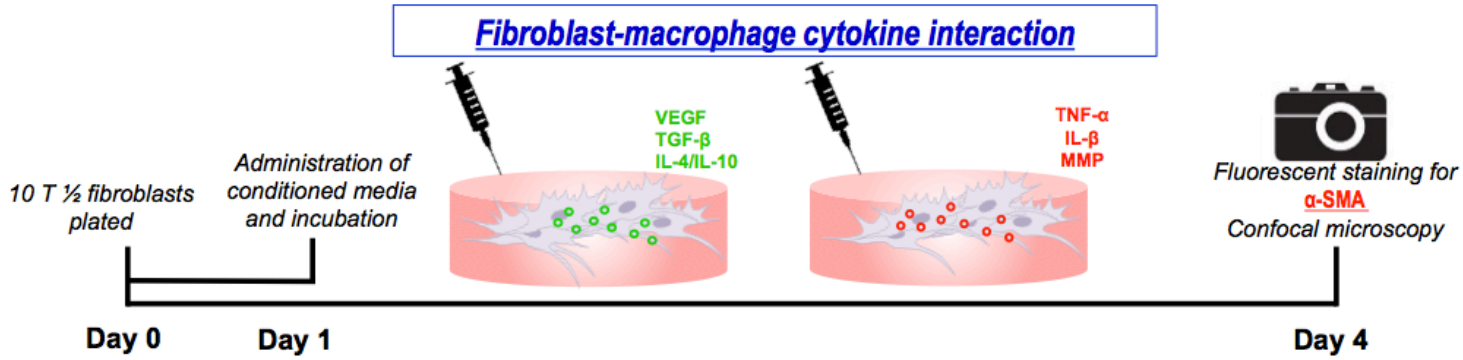


Figure 2: Conditioned Media In Vitro Experiment Timeline

Contact Dependent In Vitro Experimentation

For the contact dependent experiment (i.e. macrophage-fibroblast co-culturing), macrophages were plated in 6 well plates at a concentration of 500,000 cells per well and were polarized as described above. The seeding density chosen was on the lower end to minimize potential overgrowth of macrophages in the well and maintain high cell viability. Macrophages from the M1, M2a, and M2c phenotypes were separately extracted and resuspended 24 hours after polarization, and instead of plating the 10 T ½ fibroblasts to an 8-well plate 24 hours before adding macrophage-conditioned media, macrophages and fibroblasts were simultaneously plated, with gelatin added to each well beforehand as described earlier. Each well was administered 250 μL of 10 T1/2 media and 250 μL of macrophage media (either M1, M2a, or M2c), with a 4:1 (10,000:2,500 per well) ratio of fibroblasts to macrophages, excluding the control. The 8-well plate was incubated for 72 hours at 37 degrees Celsius.

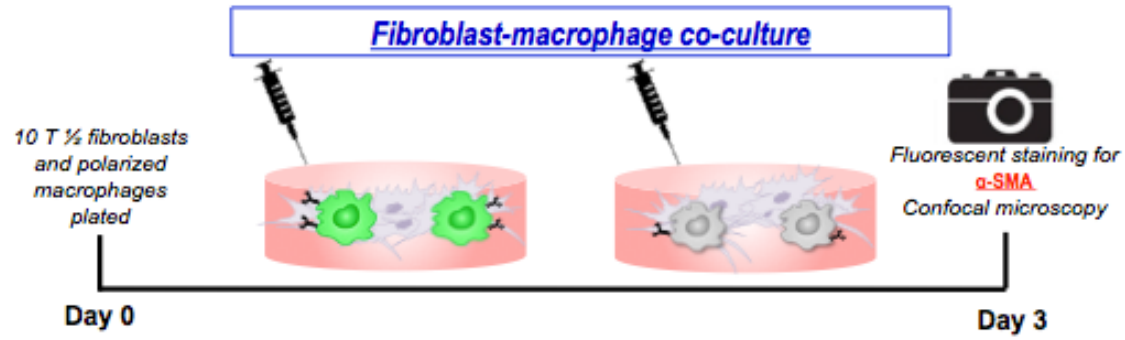


Figure 3: Contact Dependent In Vitro Experiment Timeline

Immunofluorescent Staining

For both co-culturing and conditioned media experiments, cells were stained for Phalloidin-AF88 (green channel; fibroblasts; ThermoFisher Scientific A12379) α-SMA -Cy3 (red channel; Sigma-Aldrich® C6198) and Hoescht 333-42 (blue channel; nuclei; Life Technologies™). Media was aspirated off each well and each well was washed with Dulbecco's Phosphate-Buffered Saline (DPBS; Corning™) 3 times. Cells were then fixed to the wells with 4% paraformaldehyde (PFA) solution incubated at 37 degrees Celsius for ten minutes. A 0.1 % Triton-X 100 (Sigma Aldrich) solution diluted in DPBS was then used to permeabilize the cells for staining at room temperature for 5 minutes. After permeabilization, cells were blocked with a 1% bovine serum albumin (BSA; Fisher BioReagents™) solution at 37 degrees Celsius for 20 minutes. A solution containing α-SMA -Cy3 antibody at a 1:500 dilution and Phalloidin-AF488 antibody at a 1:41 dilution in 1% BSA was administered to each well, and cells were incubated in this solution at 37 degrees Celsius for 20 minutes. Cells were then incubated in a Hoescht solution diluted 1:10,000 in standard Phosphate-Buffered Saline (PBS) under foil for 10 minutes at room temperature to stain for nuclei. After Hoescht staining, cells were washed with DPBS and

mounted using Vectamount Mounting Media™. Cells were also washed with DPBS 3 times between each step, and 250 μL of solution per well was administered per step.

Image Analysis

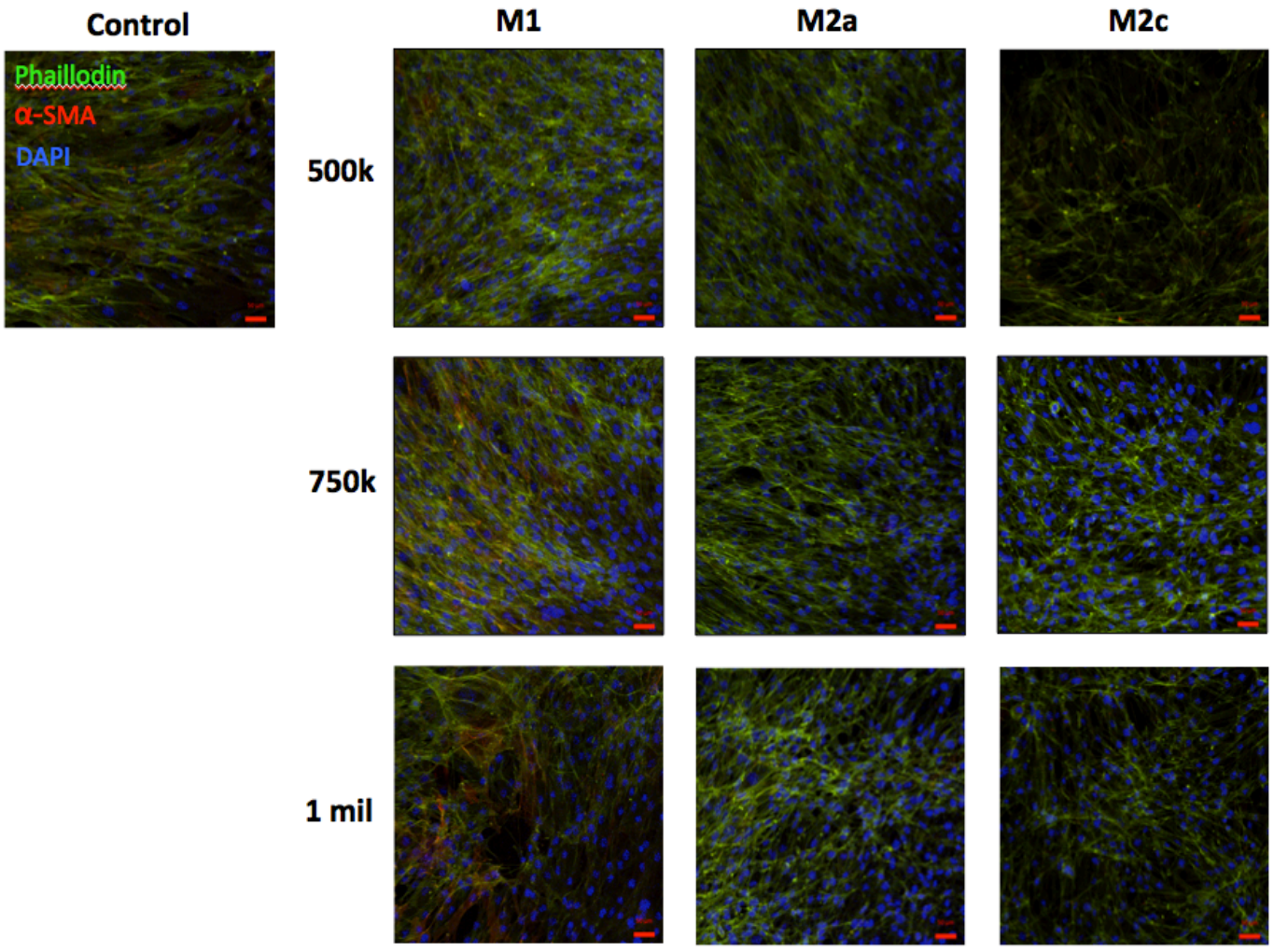
After staining, a maximum of 3 images per well were taken using a Zeiss confocal microscope and Zen Blue 2012 software with an EC-Plan Neuroflar 10x/0.3 objective. Each image had all 3 three channels (green at 488 nm, red at 555 nm, blue at 405 nm) merged to show full staining. The parameters for the red and green channel (α-SMA, phailloidin) were fixed for all images to highlight relative difference in intensity across different wells.

Images taken via confocal microscopy were analyzed for intensity of α-SMA and phailloidin via ImageJ. Each image was exported from Zen Blue 2012, separated by channel color, and converted to 8-bit. The ratio of α-SMA percent area to phalloidin percent area was calculated for each image to normalize for the fibroblast presence in each image, and a one-way ANOVA as well as a multiple comparisons test with an alpha of 0.05 was used to determine significance between phenotypic groups for each experiment.

CHAPTER 4

RESULTS

Cytokines From Different Macrophage Phenotypes Affect Fibroblast α-SMA Expression



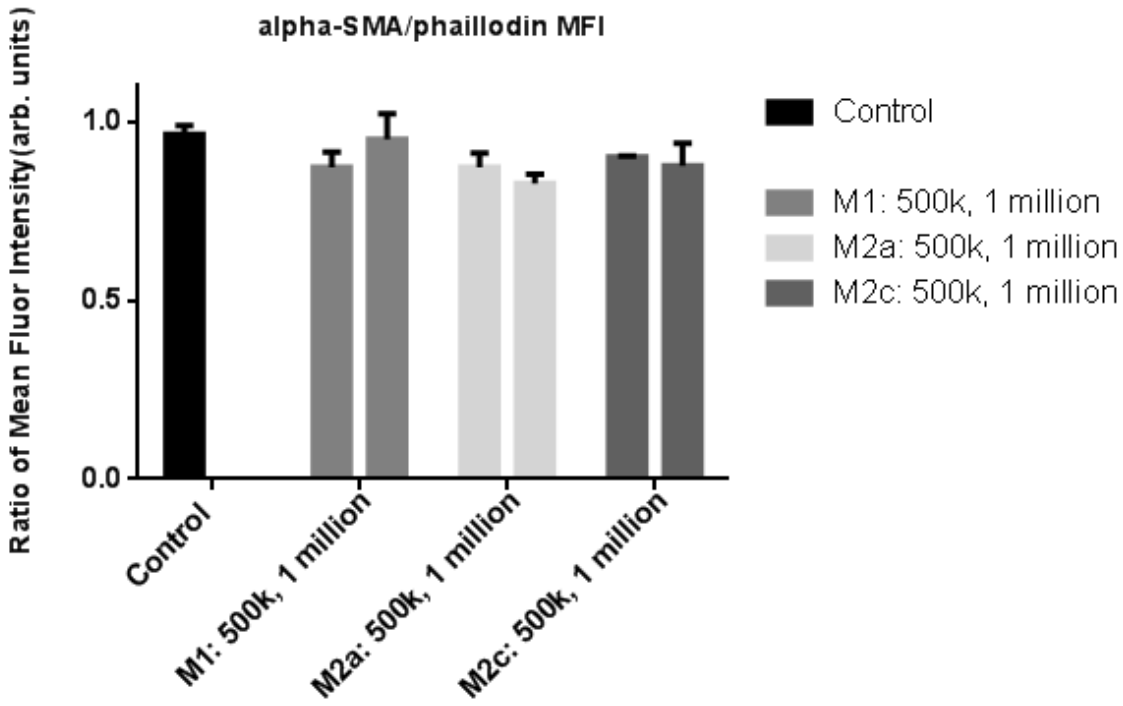


Figure 4: Effect of Phenotype and Seeding Density on Normalized α-SMA Expression.

Cytokine cocktails derived from conditioning media with different macrophage phenotypes as well as seeding densities was administered to 10 T ½ fibroblasts over a period of 72 hours. Interestingly, no administration of macrophage cytokines showed relatively high normalized α-SMA expression in comparison to the other phenotypes, regardless of seeding density. The normalized α-SMA expression for M1, M2a, and M2c groups with 500,000 macrophage seeding was similar, while M1 was higher for both 750,000 and 1 million macrophage seeding. However, no significant difference in normalized α-SMA expression was found between the phenotypes for the 500,000 and 1 million seeding density groups. Furthermore, changing the seeding density of macrophages within the range of 500,000 to 1 million for cytokine production yielded no

significant difference in normalized α-SMA expression within phenotypes. Error bars denote standard error.

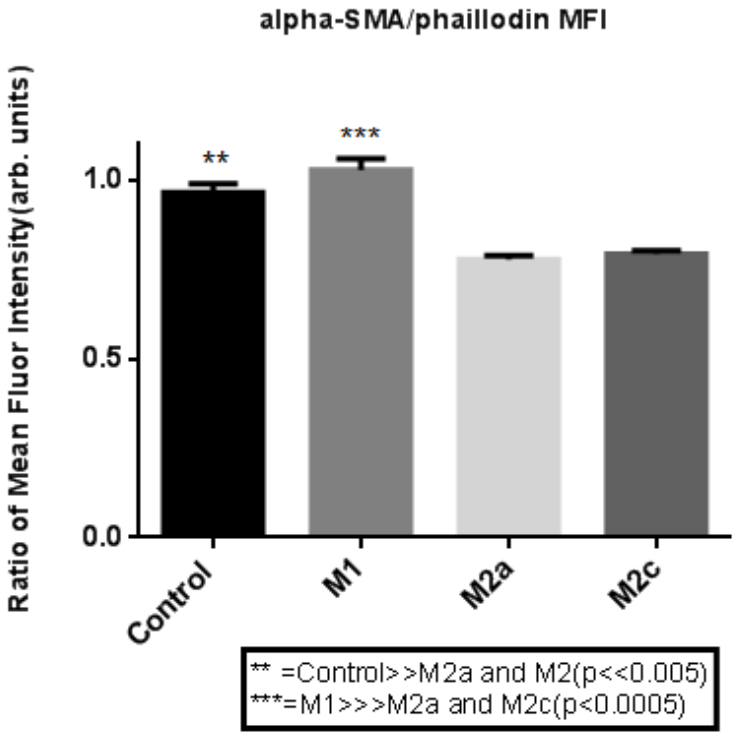
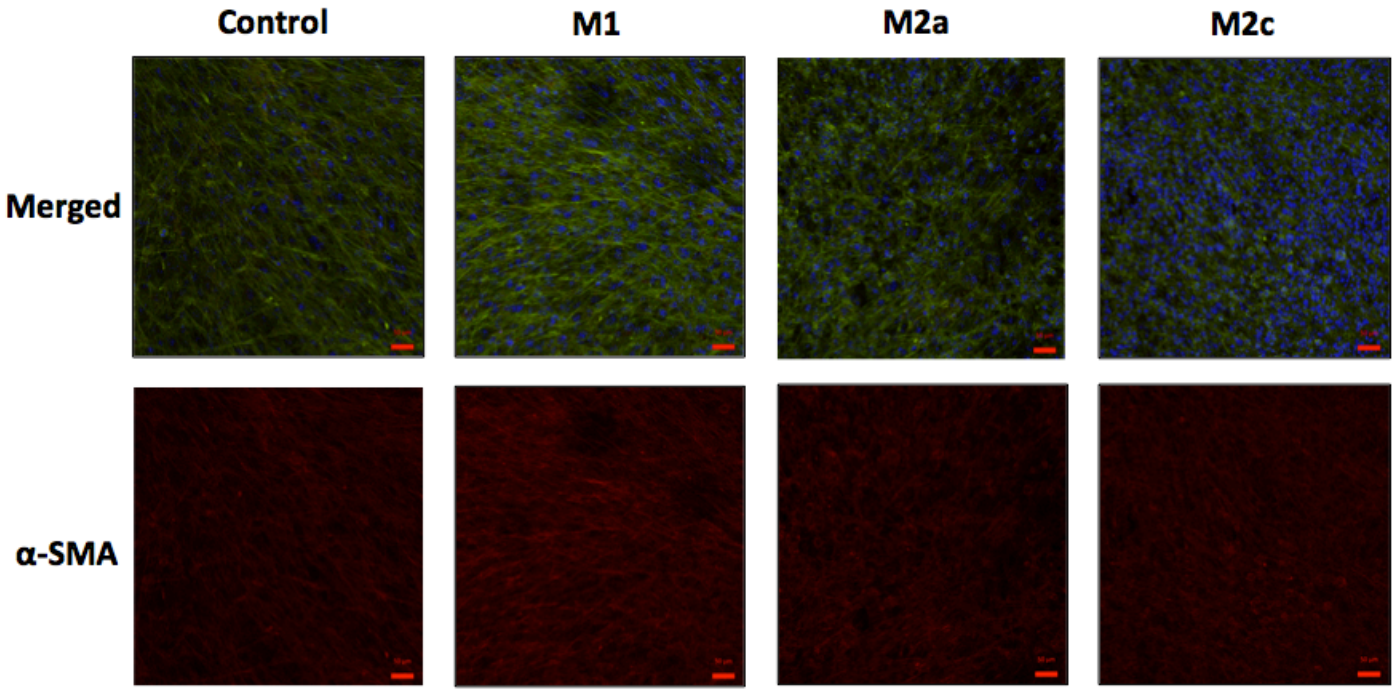


Figure 5: Macrophage Cytokine Effect on α-SMA Expression, 750,000 Seeding Density. Based on confocal images, cell adherence was lower and generally most sparse for the 500,000 and 1 million seeding groups compared to the 750,000 group.
It was determined that, for the 750,000 seeding density group, not only was the normalized α-SMA expression for M2a and M2c group significantly lower than M1 group expression, but there was also a significant decrease relative to the control. Error bars denote standard error.

Macrophage Contact Dependent Mechanisms Have No Effect on α-SMA Expression



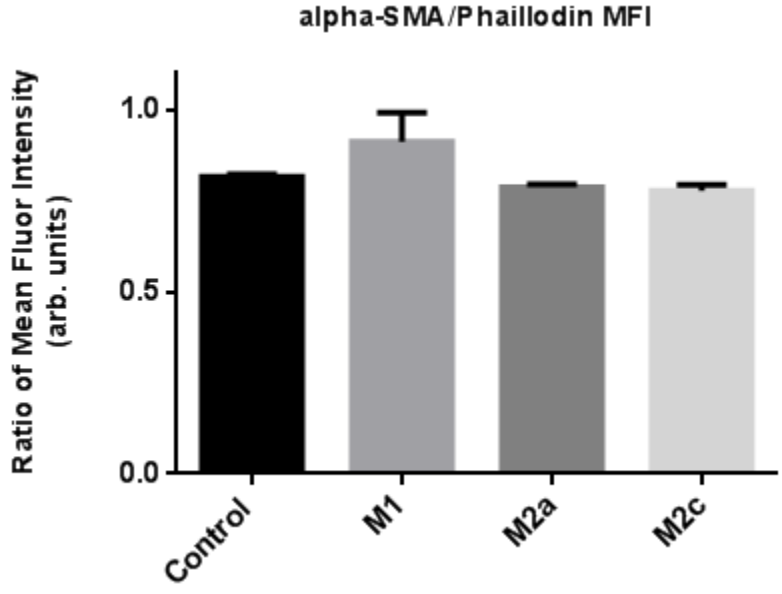


Figure 6: Macrophage Co-culture Effect on α-SMA Expression. The data for the co-culture experiment was analyzed the same as for the conditioned media experiment. Data for normalized raw integrated density (ratio of α-SMA to phalloidin) was analyzed for significance using one-way ANOVA with multiple comparisons, specifically quantifying the significance (at alpha=0.05) between means of each group. However, despite an apparent increase in average normalized α-SMA expression for the M1 co-culture group, there was not a single significant difference between groups. Error bars, again, represent standard error.

CHAPTER 5

DISCUSSION

Overview

The results of the conditioned media experiment may suggest a seeding density between (but not including) 500,000 and 1 million cells is more optimal for macrophage polarization and accompanying cytokine production using the above protocol, with fibroblasts in the 750,000 groups showing the most attachment and having the most cell dense regions. These results also show that fibroblast interacting with M1 cytokines developed within that seeding density range expressed significantly higher levels of α-SMA expression relative to both M2a and M2c, while fibroblasts incubated with M2a and M2c cytokine cocktails also had significantly lower α-SMA expression levels relative to the control group. Furthermore, in the case of contact dependency (co-culturing), there was not a single significant difference determined amongst groups.

Assessing Specificity and Cell Viability

A consistency among all groups is the apparent overlap of α-SMA and phalloidin. α-SMA stains should stain for actin filaments, although in some groups, specific filamentous regions were hard to distinguish if, in fact, they were present. The groups with most notable filamentous staining were the conditioned media control group and M1 conditioned media groups. However, there appeared to be non-filamentous staining for these groups as well. This reinforces the impetus for using a ratio of α-SMA to phalloidin as a metric to compare rates of expression, since this generally accounts for varying levels of apparent α-SMA expression that depend on fibroblast presence, and thus phalloidin staining.

In the case of the contact dependent experiment, an earlier argument was made as to why the seeding density was lower for plating and polarizing the macrophages. A significant problem in developing these experiments is ensuring that, at the end of

macrophage conditioned media or cell preparation, the macrophages have not become overpopulated and apoptotic/necrotic. For conditioned media experiments, this overgrowth and subsequent death of macrophages may lead to less cytokine production. On the other hand, if, in the 24 hours conditioning step, macrophages are at such a confluence to where they deplete the media of most nutrients while secreting cytokines, this may complicate fibroblast adhesion and proliferation. It appears that the 750,000 seeding density group may provide the best balance between overgrowth/cell death and depletion of media, however this needs to be investigated further. Necrotic factors may also exist in the media after conditioning that provide negative signals for fibroblast growth and development. Having a high viability of cells after polarization is optimal for co-culture plating. In the co-culture experiment for this study, extracted M2a and M2c macrophages were above 80% viability, whereas M1 cells were approximately around 30-40%. The lack of cell viability in M1-extracted cells could be due to the toxicity of LPS, despite its dilution.

Assessing Contact Dependency

The lack of significance in the contact dependent experiment is an intriguing find. This could be explained by the 500,000 macrophage cell seeding density, since there was no difference in normalized α-SMA expression among phenotypes in the conditioned media experiment for that group as well. However, previous literature has suggested that different signaling is involved in contact dependency compared to just exposure to cytokines in conditioned media. Another possible explanation is that contact dependent signaling between macrophages and fibroblasts actually cause fibroblasts to limit α-SMA expression, and, therefore, myofibroblast differentiation and fibrotic development. Not only does the contact dependent interaction send signals from macrophages to fibroblasts, but fibroblasts also send signals to macrophages which may provide a feedback loop affecting macrophage characterization and signaling. This explanation does follow

previous literature suggesting that macrophages presence enhances regeneration and limits fibrotic activity.

The difference in the control groups between experiments is also interesting. This difference may suggest variability introduced to the experiments which may limit comprehensive understanding of the results, at least more so in the case of the co-culture experiment.

In addition, more thorough data collection and analysis would provide edification of the above results. While normalizing α-SMA to phaillodin accounts for general fibroblast presence relative to α-SMA expression, providing a normalization relative to the amount of DAPI to determine fluorescence per cell would have provided further verification. Lastly, with the variability in macrophage cell viability during the polarization and conditioning process, the level of cytokines produced per phenotype in the experiments is uncertain. It would have been advantageous to compare the factors present in media before and after the two experiments were carried out, showing a quantifiable difference in the mechanisms at play as well as providing a check of the study's validity.

In Vivo Implications

In vivo, macrophages are more dynamic and will often switch from M1 to M2 as the wound healing stage progresses. Examining each macrophage's effect on fibroblasts may only describes isolated interactions in wound healing and not accurately illustrate the more dynamic process in vivo. However, the fact that cytokines from M1, an inflammatory macrophage, promotes higher levels of fibrotic activity in isolation compared to the M2 anti-inflammatory cytokines may suggest that anti-inflammatory cues may limit collagen development (a similar conclusion found for FTY720 therapeutic delivery in vivo) while M1 inflammatory cues may promote it.

Another intriguing find from this study is the similar levels of α-SMA expression for the M2a and M2c groups in both experiments. While some literature suggests

delineation between wound healing (M2a) and regulatory (M2c) macrophages, the differences in these subsets of M2 macrophages have not been thoroughly established. M2a and M2c may provide distinct functionality in other interactions that occur in wound healing aside from myofibroblast activation.

While macrophages and specific factors may play a role in myofibroblast development and subsequent fibrotic development, wound healing not only has fluidity in macrophage development and activity, but also encompasses a plethora of agents that influence repair and remodeling besides monocytes/macrophages. In the context of skeletal muscle, the injury site is infiltrated by satellite cells, adipose tissue, platelets, endothelial cells, and connective tissue (in addition to type I collagen scaffolding) (Järvinen et al., 2013). It is important to keep in mind the significance of the macrophage-myofibroblast activation relationship relative to the holistic process involved.

CHAPTER 6

CONCLUSION

Through an in vitro experiment culturing fibroblasts with media containing macrophage cytokines, it was determined that when macrophage cytokines were developed from a seeding density of 750,000 per well, cytokines from M2a and M2c induced a significantly lower amount of myofibroblast activation compared to the M1 and control groups. It was also found that seeding at a density of 750,000 macrophages per well showed the best cell attachment and morphology across groups. However, there was no significant difference between phenotypes in the 500,000 and 1 million seeding density groups, nor was there a significant difference in myofibroblast activation between seeding densities in each phenotype. When fibroblasts and macrophages were co-cultured with a macrophage seeding density at 500,000 originally, there was no significant difference found between phenotypes. Anti-inflammatory cues may be preferential in the context of proper remodeling and limitation of scarring, although the translation to the more dynamic in vivo process, involving M1 and M2 macrophages in sequence, remains uncertain. More investigation is necessary to comprehensively understand macrophage-fibroblast/myofibroblast interplay in wound healing.

CHAPTER 7

FUTURE WORK

In addition to verifying repeatability of the above studies, future experiments would focus on in vitro systems, including non-contact dependent reciprocal signaling via exchange of factors, where macrophages and fibroblasts are separated by a mesh. Another model which allows for M1 macrophages to transition into M2 macrophagessmay better reflect the progression of the wound healing environment, perhaps incorporating extracellular scaffolding such as laminin or fibronectin (which may also be beneficial for inclusion in the previous studies rather than temporarily adding gelatin for adhesion) as well as a skeletal muscle cell/tissue environment which would elicit the transition. There also would be a focus on establishing a protocol for optimal seeding of macrophages to develop these in vitro systems, including seeding macrophages at a density between 500,000 to 1 million cells per well in a 6-well plate. Lastly, staining for pro-collagen using models from this study may further elucidate the interplay between immunomodulation, myofibroblasts, and collagen development/tissue remodeling.

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